ISOLATION AND ANTIBACTERIAL ACTIVITY TEST OF ASSOCIATED BACTERIA FROM MARINE SPONGE *Tetilla* sp.

ISOLASI DAN UJI AKTIVITAS ANTIBAKTERI DARI BAKTERI YANG BERASOSIASI DENGAN SPONS LAUT Tetilla sp.

Dian Pribadi Perkasa[*] and Arie Budiyarto[**]

[*]Center for the Application of Isotope and Radiation Technology,
National Nuclear Energy Agency, Lebak Bulus Raya street No. 49 Jakarta Indonesia 14070
[**]Office of Environmental Impact Control, Kulonprogo Regency, Yogyakarta Indonesia
Pos-el: dpribadi@batan.go.id

ABSTRAK

Studi mengenai isolasi dan aktivitas antibakteri isolat bakteri yang berasosiasi dengan sponge laut Tetilla sp. dari pesisir Gunung Kidul telah dilakukan. Sebanyak 16 strain bakteri berhasil diisolasi, empat di antaranya mempunyai kemampuan memproduksi senyawa antibakteri. Ekstrak sel dari empat isolat bakteri tersebut diujikan aktivitas antibakteri terhadap Escherichia coli dan Staphylococcus aureus menggunakan metode difusi agar cakram kertas. Ekstrak sel keempat isolat bakteri dalam kultur cair pada fase eksponensial akhir dan fase stasioner menunjukkan aktivitas antibakteri terhadap Escherichia coli dan Staphylococcus aureus. Analisis kromatografi lapis tipis menampilkan bahwa ekstrak sel-sel pada fase stasioner mengandung terpenoid dan alkaloid, akan tetapi ekstrak sel pada fase eksponensial akhir tidak terdeteksi adanya kedua senyawa tersebut.

Kata Kunci: *Sel, Ekstrak, Bakteri, Antibakteri, Tetilla.*

ABSTRACT

The marine sponge of *Tetilla sp*. collected near Gunung Kidul coast was studied for isolation of associated bacterial and its antibacterial activity. Sixteen bacterial strains were isolated from sponge *Tetilla sp*., four isolates among it showed ability to produce antibacterial metabolite. Cell extract of isolates were tested for antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* using disc diffusion agar method. Cells extract of four selected isolate from late exponential phase and stationary phase broth culture showed antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Thin layer chromatography analysis detected alkaloid and terpenoid in cell extract from stationary phase of isolates culture and none of it was detected in cell extract from late exponential phase of isolates culture.

Keywords: Cells, Extracts, Bacteria, Antibacterial, Tetilla.

INTRODUCTION

Marine sponges are rich sources of potentially valuable natural products with broad spectrum of bioactive activity such as antibacterial, antifungal, antiviral, and inflammatory activities.[1,2,3,4] The majority of the natural products currently in clinical and pre-clinical trials are produced by marine invertebrates especially marine sponges.[2,5,6,7] The origin of bioactive compounds in marine sponges has been supposed to be from either sponge itself, their symbiotic microorganisms, or a cooperative interaction between sponge and symbiotic microbes.[8,9,10] *In situ* hybridization techniques for targeting biosynthesis genes of interest have been introduced and have provided strong evidence for the bacterial origin of marine

invertebrate bioactive compounds and expression of related genes for their synthesis.[11,12]

Even though, culture-dependent approach using proteomic and genomic analysis are rapidly developed, culturing associated microbes remains an important step in the process of understanding the biology and ecology of microbial species. Isolate culture can be used to obtain complete genome sequence and to identify the properties of organisms that could not be identified by genome sequence alone.[13] The increasing data regarding sponges associated bacteria will enhances the possibility to find novel compounds.[14,15,16,17]

Marine sponge *Tetilla* sp. dominates habitat of Gunung Kidul coast among eleven species sponges living in there. Species *Tetilla* sp. not yet explored for its associated bacteria.[18] The aim of this study is to isolate sponge associated bacteria from marine sponge *Tetilla* sp. and its antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*.

METHODOLOGY

The sponge *Tetilla* sp. was collected in the intertidal zone during low tide on Gunung Kidul coast, Yogyakarta. During sample collection, the sponge samples put into sterile plastic bag containing sea water. The sponge samples was immediately processed or stored in refrigerator under 20˚C.

The living sponge material was immediately rinsed with sterile seawater to remove the non-attached bacteria. A weight of 0.5 g sponge tissue was homogenized and inoculated on the surface of Marine Agar 2216 plate (Difco) and incubated at 30˚C in dark aerobic condition until visible colonies appear. All colonies with different morphology were picked out and purified by repeated single colony subculture.

For preliminary screening, each isolate was tested for ability to produce antibacterial metabolites using technique that described by Kanagasabhapathy *et al*.[19] with a modification. Pure culture of associated bacteria was inoculated into 10-ml aliquots of Nutrient Broth (Difco). At a stationary phase of growth at 30˚C, each culture was centrifuged 15,000 x *g* at 4˚C for 5 minutes. Antibacterial testing of extracts which were concentrated from supernatants 10 folds was performed by the paper disc diffusion technique.

For antibacterial testing, pure culture of associated bacteria were inoculated into 10 ml aliquots of Marine Broth 2216 (Difco) and incubated at 30˚C in dark aerobic condition. At an exponential phase of growth, each subculture of selected isolates was centrifuged 15,000 x *g* at 4˚C for 5 minutes. Crude biomass of cells and 10-fold concentrated supernatant was tested for antibacterial activity by disc diffusion agar technique as described by Helio *et al*.[20] A volume of 60 µl of cell extract was used to saturate a sterilized paper disc (Whatman, 5 mm), allowed to dry at room temperature and placed on the surface of Mueller Hinton Agar plates which had been freshly swabbed with the liquid culture of *Escherichia coli* and *Staphylococcus aureus*. Same procedure was performed to all isolate cultures from stationary phase of growth.

The bioactive metabolite in cell extract of selected isolates was detected using Thin Layer Chromatography (TLC) analysis. Bacterial cells that were lysed with methanol was added to concentrated supernatant and then extracted with ethyl acetate. Then crude extract was dried in vacuum using rotary evaporator. Then the extract was re-dissolved in methanol and analyzed by TLC on silica gel plates (silica gel 60, F254 Merck) with solvent and visualization system as described in Table 1.

RESULT AND DISCUSSION

Morphology of sponge sample and its megasclere spicules types were showed on Figure 1.

Table 1. Solvent System and Visualization for Bioactive Metabolites in TLC Analysis

TLC Analysis	Solvent system	Visualization
Alkaloid	Buthanol : Acetic Acid : Water	UV 365 nm
Terpenoid	n-heksane : ethyl acetat Toluene : Ethyl Acetate	UV 365 nm

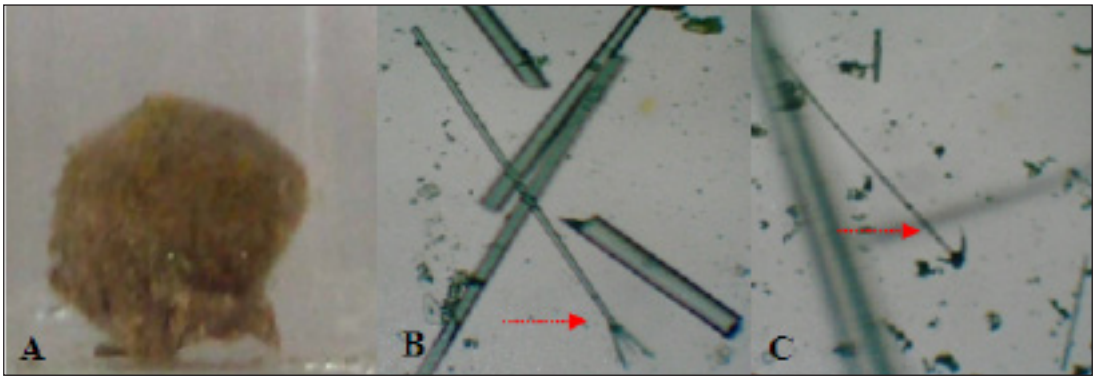

Figure 1. Morphology of *Tetilla* sp. and its specific megasclera spicules. (A) Morphology of *Tetilla* sp.; (B) Protriane shape's megasclere spicules (magnify 400X); (C) Anatriane shape's megasclere spicules (magnify 400X)

Romimohtarto and Juwana[21] described that sponge *Tetilla* sp. has yellowish brown in colour, spherical shape like golf ball and live solitaire attach to rock floor of the coast. Furthermore, sponge *Tetilla* sp. was identified based on specific protriane and anatriane shape of its megasclere spicules. Based on Figure 1, collected sponge sample was confirmed as *Tetilla sp*.

Isolation of Bacteria

Sixteen sponge associated bacterias were successfully isolated from homogenized sponge tissue on the Marine 2216 agar plate. Bacterial isolates were differentiated based on morphological characteristics of colony and cell (showed in Table 2).

Sixteen numbers of bacteria that were successful isolated in this researches believed far below from the total bacteria that associated to sponge *Tetilla* sp. Bacterial densities in sponge tissue may reach up to 10^8-10^{10} cells bacteria per gram of sponge wet weight.[22] The difference in order of magnitude between colonies on agar media and the numbers countable by microscopic examination was known as the great plate count anomaly.[23] There is only 0.01 to 0.1% of oceanic marine bacterial cells produces colonies by standard plating technique.[24,25] Deming and Baross[26] explained that unculturable isolates may fail to grow because their growth state in nature prevents the adjustment to condition found in the medium used for the plate counts. It also explained that many of the microbial species that dominant in natural setting are not adapted for growth in media containing high concentration of carbon organic carbon.[27] Specialized techniques such as high-throughput method in very low nutrient media can be applied to improve the numbers of isolated microbial up to 14% of total microbes.[13]

Growth of Bacteria

Six of sixteen isolates showed the ability to produced antibacterial metabolite in preliminary screening and four isolates were selected to be cultivated for further test. Four selected isolates were isolates BHST2, BHST5, BHST6, and BHST15 gave clear inhibitory zone around paper disc. Isolates BHST2 showed clear inhibition zone but in a narrow area, and isolates BHST16 only showed cloudy inhibitory zone around paper disc.

Cultivation of four selected isolates was using Marine Broth 2216 medium. Growth curve of the selected isolates in Marine Broth 2216 showed in Figure 2.

Based on Figure 2, broth culture were collected after 16 hours incubation for bacterial cells at late stationary phase and after 20 hours incubation for bacterial cells at stationary phase. This growth curve was used to determine incubation time needed by isolate culture to reach late exponential phase and stationary phase.

Four selective isolates were categorized as fast growing bacteria. Isolates growth easily in Marine Broth 2216 medium and reached stationary phase only within 20 hours. A research conducted by Zheng *et al.*[28] stated that to incubate marine bacteria in broth culture needed for 7 days to reach stationary phase. This different

Table 2. Morphology Characteristics of Associated Bacteria Isolates from Marine Sponge *Tetilla* sp.

Isolates	Morphology Characteristics						Ability to produce Antibacterial Metabolites	
	Colony				Cell			
	Shape	Elevation	Edge	Inner structure	Shape	Gram staining	*Escherichia coli*	*Staphylococcus aureus*
BHST1	circular	effuse	lacerate	smooth	long rod	positive	positive	negative
BHST2	circular	effuse	lacerate	smooth	coccus	negative	positive	positive
BHST3	circular	low confex	undulate	smooth	short rod	positive	negative	positive
BHST4	circular	low confex	lacerate	opaque	short rod	positive	negative	negative
BHST5	myceloid	effuse	undulate	smooth	triangle	negative	positive	positive
BHST6	myceloid	low confex	undulate	fin granular	coccus	positive	positive	positive
BHST7	circular	low confex	entire	smooth	short rod	negative	negative	negative
BHST8	circular	effuse	entire	opaque	long rod	negative	negative	negative
BHST9	myceloid	effuse	lacerate	smooth	long rod	positive	negative	negative
BHST10	circular	effuse	entire	smooth	coccus	positive	negative	negative
BHST11	circular	low confex	entire	opaque	short rod	positive	negative	negative
BHST12	circular	effuse	lacerate	opaque	coccus	negative	negative	negative
BHST13	circular	low confex	undulate	fin granular	long rod	negative	negative	negative
BHST14	circular	effuse	entire	smooth	long rod	negative	negative	negative
BHST15	circular	convex	entire	opaque	short rod	negative	positive	negative
BHST16	circular	effuse	undulate	Translucent	Coccus	negative	positive	negative

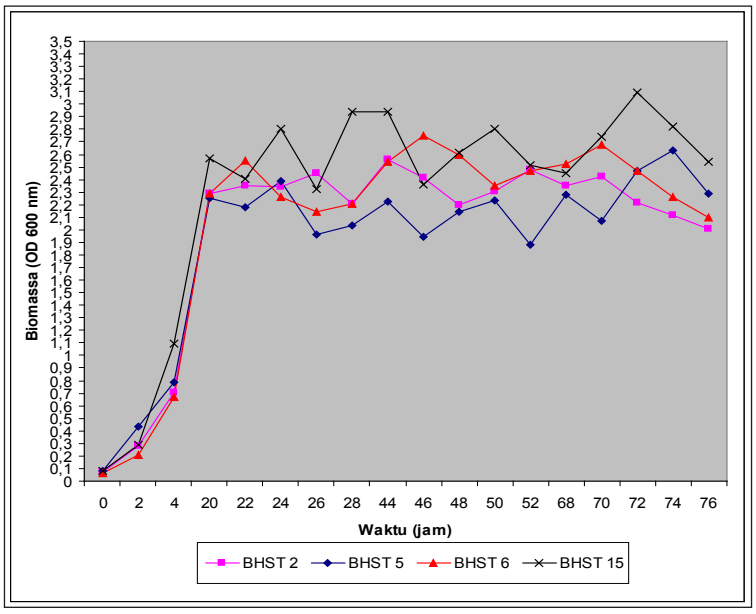

Figure 2. Growth curve of four selected isolates in Marine Broth 2216

incubation time may be caused by different growth characteristic of isolates whereas isolates of Zheng *et al.*[28] were categorized as fastidious oligotrophic bacteria and isolates in this research were categorized as heterotrophic bacteria. Based on Vancanneyt *et al.*[27], sponge associated bacteria categorized as heterotrophic bacteria can easily isolated and cultivated without special technique just as conducted in this research.

Antibacterial Activity

Antibacterial activity of cell extract from late exponential and stationary phase of isolates culture against *Escherichia coli* and *Staphylococcus aureus* and its TLC analysis showed in Table 3 and Table 4.

Based on Table 3, antibacterial metabolites of cell extract from late exponential phase culture of isolates showed different antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Each isolates will produce different type of bioactive metabolites with different activity. Some isolates may produce narrow spectrum antibacterial activity, such as isolate BHST6 that only inhibit growth of *Staphylococcus aureus*.[29] Isolates BHST2 and BHST5 showed broad spectrum antibacterial activity by inhibited growth of both *Escherichia coli* and *Staphylococcus aureus*. Antibacterial activity against *Escherichia coli* was lower than *Staphylococcus aureus*. It is presume that complex structure of Gram negative bacteria cell wall may give more protection from bioactive metabolites of those isolates.[30]

Production of bioactive metabolite in exponential phase may be caused by supression of transition state regulator gene to stationary phase gene. Beside causing production of bioactive metabolite, this condition also lead to cell differentiation and inhibition to the growth of isolates. This pattern showed in Figure 2 where as isolates BHST15 growth faster than other isolates that produce bioactive metabolites in late exponential phase. Production of bioactive matabolite in exponential phase ussually avoided in bioreactor.[31]

Based on Table 4, antibacterial metabolites of the cell extract from stationary phase also showed different antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Comparing to the cell extract from late exponential phase culture, the cell extract from stationary phase culture showed higher antibacterial activity. It Seemed that amount and diversity of bioactive metabolite in an isolate culture will be higher after longer incubation time.[31]

Alkaloid and terpenoid were detected only in bioactive metabolite from stationary phase culture based on TLC analysis. Moore[32] summarize in a review that biosynthesis of almost all marine natural product by microorganism started when cell culture at stationary phase. This condition explained why cell extract of isolate BHST15 from late exponential phase showed no antibacterial activity of but show a good antibacterial activity of bioactive metabolite from stationary phase.

Table 3. Antibacterial Activity of Cell Extract from Late Exponential Phase Isolate Culture and Its TLC Analysis

Isolates	Antibacterial activity against		TLC analysis
	Escherichia coli	*Staphylococcus aureus*	
BHST-2	*	**	-
BHST-5	*	**	-
BHST-6	-	*	-
BHST-15	-	-	-

*diameter of inhibition less than 9 mm; **diameter of inhibition 9-13 mm.

Table 4. Antibacterial Activity of Cell Extract from Stationary Phase Isolate Culture and Its TLC analysis

Isolates	Antibacterial activity against		TLC analysis
	Escherichia coli	*Staphylococcus aureus*	
BHST-2	**	***	Terpenoid
BHST-5	**	***	Alkaloid and terpenoid
BHST-6	-	***	Alkaloid and terpenoid
BHST-15	***	-	Terpenoid

*diameter of inhibition less than 9 mm; **diameter of inhibition 9-12 mm;*** diameter of inhibition more than 13 mm.

Antibacterial activity of sponge *Tetilla* sp. associated bacteria in this research are relatively equal compare to antibacterial activity of surface isolated bacteria from the marine sponge *Pseudoceratina purpurea* in Mandapam coast, Gulf of Mannar, India.[19] Based on definition in this research, antibacterial activity of sponge *Tetilla* sp. was defined as mild, moderate and strong activities as inhibition zone of < 9 mm, 9–12 mm, and > 5 mm.

It is interested to discuss about bioactive metabolite of isolate BHST2, BHST5, and BHST6 from late exponential phase. Recent research found that secondary metabolite such as polyketide and non ribosomal peptide synthesis (NRPS) are produced in exponential phase.[33] Based on carbon/chemical balance hypothesis of chemical defend theory, environmental change that lead to carbohydrate status will lead to parallel changes in levels of carbon-based secondary metabolites. Overflow metabolism of isolate in rich nutrient medium can generate transformation of excessed primary metabolites to be secondary metabolites.[34] As polyketide was produced via lipid biosynthesis pathway and NRPS included as protein based metabolites,[35] it is possible to hipothesized that bioactive metabolites in cell extract from late exponential phase are polyketide and/or NSRP.

CONCLUSION

This study demonstrated that four of sixteen sponge *Tetilla* sp. associated bacteria from Gunung Kidul coast, Yogyakarta showed mild to strong antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. Advance technique using molecular biology and nuclear technology interesting to be applied to give better knowledge and biotechnological opportunities about biosynthesis of bioactive metabolite by sponge associated bacteria.

ACKNOWLEDGEMENTS

We thank to Prof. Dra. Endang Sutariningsih Soetarto, M.Sc., Ph.D. and Prof. Dr. Sukarti Moeljopawiro, M. App.Sc. for their help and valuable suggestions.

REFERENCES

[1]Friedrich A.B., *et al.* 2001. Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol Ecol,* 38: 105–113.

[2]Proksch, P., R.A. Edrada and R. Ebel. 2002. Drugs from the seas: current status and microbiological implications. *Appl Microbiol Biotechnol,* 59: 125–134.

[3]Muller, W.E.G. and I. M. Muller. 2003. Analysis of the sponge (Porifera) gene repertoire: implications for the evolution of the metazoan body plan. In W.E.G. Muller (Ed.). *Sponges (Porifera)*:1–34. Berlin: Springer.

[4]Blunt J.W., *et al*. 2007. Marine natural products. *Nat Prod Rep,* 24: 31–86.

[5]Haefner B. 2003. Drugs from the deep: marine natural products as drug candidates. *Drug Discov Today,* 8: 536–544.

[6]Newman D. J. and G. M. Cragg. 2004. Marine natural products and related compounds in clinical and advanced preclinical trials. *J Nat Prod,* 67: 1216–1238.

[7]Fenical W. 2006. Marine pharmaceuticals: past, present and future. *Oceanography,* 19: 110–119.

[8]Faulkner, D.J., *et al*. 1993. New metabolites from marine sponges – are symbionts important? *Gazzetta Chimica Italiana,* 123: 301–307.

[9]Uriz, M. J., X. Turon, J. Galera and J. M. Tur. 1996. New light on the cell location of avarol within the sponge *Dysidea avara* (Dendroceratida). *Cell Tissue Res,* 285: 519–527.

[10]Flowers, A. E., *et al*. 1998. Cellular origin of chlorinated diketopiperazines in the dictyoceratid sponge *Dysidea herbacea* (Keller). *Cell Tissue Res,* 292: 597–607.

[11]Davidson, S.K., *et al*. 2001. Evidence for the biosynthesis of bryostatins by the bacterial symbiont '*Candidatus* Endobugula sertula' of the bryozoan *Bugula neritina*. *Appl Environ Microbiol,* 67: 4531–4537.

[12]Flatt, P., J. *et al*. 2005. Identification of the cellular site of polychlorinated peptide biosynthesis in the marine sponge *Dysidea* (*Lamellodysidea*) *herbacea* and symbiotic cyanobacterium *Oscillatoria spongeliae* by CARD-FISH analysis. *Mar Biol,* 147: 761–774.

[13]Connon, S.A. and S.J. Giovannoni. 2002. High-Throughput Methods for Culturing Microorganism in Very-Low-Nutrient Media Yield Diverse New Marine Isolates. *Applied and Environmental Microbiology,* 68 (8): 3878–3885.

[14]Webster, N. S., K. J. Wilson, L. L. Blackall and R. T. Hill. 2001. Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl Environ Microbiol,* 67: 434–444.

[15]Hentschel, U., *et al*. 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol,* 68: 4431–4440.

[16]Kim, T. K., M. J. Garson and J. A. Fuerst. 2005. Marine actinomycetes related to the '*Salinospora*' group from the Great Barrier Reef sponge *Pseudoceratina clavata*. *Environ Microbiol,* 7: 509–518.

[17]Montalvo, N.F., N.M. Mohamed, J. J. Enticknap and R.T. Hill. 2005. Novel actinobacteria from marine sponges. *Antonie Van Leeuwenhoek,* 87: 29–36.

[18]Triyanto, A. 2003. *Keanekaragaman Jenis sponge (Porifera: Demospongia) di Pantai Krakal Gunung Kidul DIY*. Seminar, Faculty of Biologi. Yogyakarta: Gadjah Mada University.

[19]Kanagasabhapathy, M., *et al*. 2005. Inhibitory Activities of Surface Associated Bacteria Isolated from the Marine Sponge Pseudoceratina purpurea. *Microbes and Environment,* 20 (3): 178–185.

[20]Hellio C., *et al*. 2001. Inhibition of marine bacteria by extract of macroalgae: potential use for environmentally friendly antifouling paints. *Mar Environ Res,* 52: 231–247.

[21]Romimohtarto K., and Juwana S. 2001. *Biologi Laut: Ilmu Pengetahuan tentang Biota Laut*. Jakarta: Penerbit Djambatan.

[22]Hentschel U., K.M. Usher and M.W. Taylor. 2006. Marine sponges as microbial fermenters. *FEMS Microbiol Ecol,* 55: 167–177.

[23]Staley, J. T. and A. Kanopka. 1985. Measurements of *in situ* activities of nonphotosynthetic Microorganism in Aquatic and Terestrial Habitats. *Ann Rev Microbiol,* 39: 3321–346.

[24]Kogure K., U. Simidu and N. Taga. 1979. A Tentative Direct Microscopic Method for Counting Living Marine Bacteria. *Can J Microbiol,* 25: 415–420.

[25]Hentschel U. *et al*. 2003. Microbial diversity of marine sponges. *Prog Mol Subcell Biol,* 37: 59–88.

[26]Deming, J.W. and J.A. Baross. 2000. Survival, Dormancy and Nonculturable Cells in Extreme Deep-Sea Environment. In R. R. Colwell and D. J. Grimes (Ed.). *Nonculturable Microorganism in the Environment*:147–197. Washington D. C.: ASM Press.

[27]Vancanneyt, M., *et al*. 2002. Characterization of an Autothropic Sulfide-Oxidizing Marine *Arcobactor* sp. that Produces Filamentous Sulfur. *Appl Environ Microbiol,* 68: 316–325.

[28]Zheng, L., *et al*. 2005. Marine Bacteria Associated with marine microorganism: the Potential Antimicrobial Resources. *Annals of Microbiology,* 55 (2): 119–124.

[29]Prescott, L.M., J.P. Harley and D.A. Klein. 2002. *Microbiology*. New York: McGraw-Hill.

[30]Madigan, M.T., J.M. Martinko and J. Parker. 1997. *Brock Biology of Microorganisms*. London: Prentice Hall International, Inc.

[31]Lucking, G., *et al*. 2009. Cereulide synthesis in emetic *Bacillus cereus* is controlled by the transition state regulator *AbrB*, but not by the virulence regulator *PlcR*. *Microbiology,* 155: 922–931.

[32]Moore, B.S. 2005. Biosynthesis of Marine Natural Product: Microorganism (Part A). *Nat Prod Rep,* 22: 580–593.

[33]Peiru, S., *et al*. 2005. Production of Potent Antibacterial Polyketide Erythromycin C in *Escherichia coli*. *Applied and Environmental Microbiology,* 71 (5): 2539–2547.

[34]Berenbaum, M. R. 1995. The Chemistry of Defence: Theory and Practice. In T. Weisner and J. Meinwald (Ed.) *Chemical Ecology: The Chemistry of Biotic Interaction*. Washington, D. C.: National Academy of Science.

[35]Mahmud, T., *et al*. 2002. A Novel Biosynthetic Pathway Providing Precursors for Fatty Acid Biosynthesis and Secondary Metabolites Formation in Myxobacteria. *The Journal of Bilogical Chemistry,* 227 (36): 32768–32774.